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GELATION OF AMYLOSE

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ABSTRACT

A range of physical techniques, including light-scattering, turbidity measurements, viscometry, dilatometry, rheological measurements, and X-ray diffraction, has been used to study the gelation of amylose. Gels form on cooling entangled amylose solutions and occur as a result of a phase separation which produces a three-dimensional polymer network. Crystallisation, as detected by X-ray diffraction, was observed to be a slower process originating in the polymer-rich phase.

INTRODUCTION

Starch occurs naturally as water-insoluble granules. It consists of two main polysaccharides, amylose and amylopectin, both of which are based on (1→4)-linked α-D-glucose chains. Whereas amylose is an essentially linear macromolecule, amylopectin is highly branched with branches linked at the 6 position. Amylose is usually defined[1], on the basis of its interaction with iodine in aqueous solution, as that starch polysaccharide which, under standard conditions, binds 19.5 ±0.5% w/w of iodine.

When an aqueous suspension of starch granules is heated above a certain temperature, known as the gelatinisation temperature, irreversible swelling of the granules occurs, resulting mainly in the release of amylose into solution[1]. Provided the starch concentration is high enough, this suspension behaves as a viscoelastic paste. On cooling, the paste is transformed into an opaque elastic gel. The pastes and gels may be regarded as composite materials with the swollen granules filling the polymer solution or polymer gel[2]. The changes observed after cooling the viscoelastic paste have been called retrogradation. It is generally believed[3] that the initial rheological changes accompanying starch gelation result from the gelation of the amylose matrix. Thus, studies of the gelation of amylose are relevant to an understanding of the retrogradation of starch.

Current experimental data suggest that amylose, when in dilute, neutral, aqueous solution, behaves as a flexible coil[1]. At room temperature, even dilute aqueous solutions are inherently unstable. After a limited period of time, dependent on polymer concentration and molecular weight, the amylose precipitates from solution. When concentrated solutions of amylose are cooled to room

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temperature, the samples form opaque elastic gels. Amylose which has precipitated from dilute aqueous solution yields an X-ray powder diffraction-pattern consistent with that observed for the B-type crystalline form of starch[4]. The same powder diffraction-pattern has been shown to develop upon storage of amylose gels[5]. There is still some debate about the interpretation of these diffraction patterns. Recent fibre-diffraction studies on the B-form[6] suggest that amylose packs as antiparallel double-helices in a hexagonal array. Biopolymer gels are usually formed by the physical association of parts of polymer chains at regions of local order or "junction zones" to produce a continuous, three-dimensional polymer network[7]. The gelation of amylose has been regarded as a partial crystallisation process[8] and it has been suggested that the ordered "junction zones" of the gel consist of microcrystalline regions[6,8].

Recent work has questioned this view[9]. Preliminary studies were made to establish the role of crystallisation in the development of the network structure during the gelation of amylose. Measurements of the development of turbidity and also of the network structure, as monitored by the shear modulus, showed that both processes occurred over similar time-scales. The development of crystallinity, as detected by X-ray diffraction, occurred on a longer time-scale. The results of these studies were taken to suggest that two processes contributed to the early stages of gelation (retrogradation). A rapid phase separation was envisaged, with a slower development of crystalline regions within the polymer-rich regions. We now report a continuation of these earlier preliminary studies. The initial gelation and subsequent crystallisation of amylose has been studied using a wide range of biophysical techniques.

MATERIALS AND METHODS

General methods. — Starch was extracted and purified from smooth-seeded leafless peas (var. Filby) by the aqueous extraction procedure[10]. The purified starch had an amylose content of 25% w/w, as assessed by its iodine-binding capacity, measured using a semi-micro, differential potentiometric method[11]. Polysaccharide concentrations were measured by the phenol–sulphuric acid colorimetric method[12].

Isolation and characterisation of amylose. — The gelatinisation temperature of the starch was 62°. Amylose was prepared by aqueous leaching of the granules under nitrogen in the temperature range 62–70°. The swollen, gelatinised granules were removed by centrifugation (~2000*g*) followed by filtration through glass sinters (porosity 2). The amylose was precipitated by the addition of 1-butanol (8% w/w), and the complex was collected by centrifugation (~2000*g*). Amylose solutions were regenerated from the complex by heating to 95° followed by removal of the 1-butanol in a heated nitrogen stream. The purified amylose had an iodine-binding capacity of 19.5%. Linearity of the amylose molecules was assessed by measuring the beta-amylolysis limit, using a crystalline beta-amylase (sweet potato, Sigma). The maltose released was measured by a reducing-sugar method[13]. The absence of

alpha-amylase was demonstrated by the inability of the beta-amylase preparations to release chromophore as low-molecular-weight-fragments from amylose–azure preparations. Also, the reducing power of maltose solutions showed no detectable increase on incubation with the beta-amylase preparation, indicating the absence of α-D-glucosidase. The amylose preparation was quantitatively converted into maltose by the beta-amylase, indicating that it was a linear amylose with no detectable amylopectin present.

The molecular weight and size of the amylose was determined by light scattering. Hot dilute solutions of amylose were cooled to room temperature, filtered through 0.22-μm cellulose acetate filters, and diluted with filtered water to the required concentration. Light-scattering studies were carried out within 2–3 h of preparation. No systematic changes in scattered intensity were observed during these studies, suggesting the absence of retrogradation. However, on storage for ~12 h, extensive precipitation was observed. The samples showed no evidence of abnormally large light-scattering intensities at low angles. On addition of alpha-amylase, the scattering envelope decreased rapidly to values virtually indistinguishable from the solvent scattering. Both tests, together with previous degradation studies with beta-amylase and measurements of iodine-binding capacity, were consistent with true molecular solutions and indicate the absence of detectable amylopectin and retrograded amylose.

Light-scattering measurements were made with a modified Malvern 4300 spectrometer system, using vertically polarised light (442 nm). Measurements of the Rayleigh ratio were made relative to benzene, and the Rayleigh ratio for benzene was taken[14] to be $R_{90°} = 64.23 \times 10^{-6} m^{-1}$. Depolarisation corrections[14] were applied for benzene samples, but were considered to be negligible for amylose samples. The specific refractive index increment at 442 nm was taken to be 0.15 $mL.g^{-1}$ for aqueous amylose solutions[15]. Data were analysed by the Zimm-plot method[16]. Further details of the light-scattering studies and the combined use of static and dynamic light-scattering methods to assess polydispersity will be published elsewhere[17].

Viscometry. — The dependence of specific viscosity on concentration, over a wide range of concentrations, was measured using Ubbelohde suspended-level viscometers. To prevent retrogradation occurring, these studies were carried out at 65°.

Turbidity measurements. — The development of turbidity with time on gelation of amylose was monitored by using a Pye Unicam SP800 spectrophotometer (640 nm, 1-cm pathlength). The turbidity of the solutions and gels was measured relative to water.

Rheological measurements. — Network formation was monitored by following the development of the shear modulus G′. Values of G′ were determined by using a modified version of a Rank Brothers' Pulse Shearometer. This apparatus is based on a design originally due to van Olphen[18] and subsequently modified[19]. Samples were contained between two perspex discs positioned at a distance "l"

apart. The upper disc was made to oscillate through a small angle ($\simeq 1 \times 10^{-4}$ radian) by the application of a square-wave pulse to a piezo-electric crystal coupled to the perspex disc. A shear-wave (frequency, 200 Hz) was transmitted through the sample and detected by the lower perspex disc. The resultant twist was detected by a second piezo-electric crystal and output as an electrical potential difference. From the time taken to traverse the distance "l", the velocity of the sound wave (V) may be calculated. Then

$$G' = pV^2 (1 - r^2)/(1 + r^2)^2,$$

where p is the density of the medium, $r = (\alpha\lambda)/2\pi$, λ is the wavelength of the sound wave in the medium, and α is the attenuation coefficient. For amylose gels, the damping of the shear-wave is small and the correction factor $(1 - r^2)/(1 + r^2)^2 \approx 1$. It is normal practice to time the shear-wave for various plate separations. From a plot of propagation time against plate separation, the sound velocity may be determined. This eliminates the need to measure the dead time of the apparatus (*i.e.*, the apparent elapsed time at zero plate separation). For gelation studies, it was necessary to contain the initially fluid samples in an outer perspex cylinder and to work with a fixed plate separation. Variations in plate separation would destroy the weak network structure being developed. Hence, the dead time of the apparatus was determined prior to a gelation experiment. In order to quench the fluids to cause gelation, a further modification to the apparatus was made. Quenching was achieved by the passage of cold water through a large metal cooling-coil contained within the fluid and surrounding the material contained between the perspex discs.

Dilatometry. — The apparatus, which is shown in Fig. 1 and described in more detail elsewhere[20], was constructed in borosilicate glass. The capillary was precision-bore (0.2×10^{-3}-m diameter) Veridia tubing. Taps were made of poly(tetrafluoroethylene) (HP Rotaflo Quickfit and Quartz) which gave an excellent seal. The lower part of the dilatometer was filled with clean mercury. One side-arm contains water, the other the amylose solution. All solutions were carefully degassed before use and scrupulous care was taken to avoid the introduction of air bubbles. The dilatometer was mounted in a glass-fronted water bath which was capable of controlling temperature to $\pm 0.001°$ for periods of 24 h. The temperature of the bath was continuously monitored using a Hewlett–Packard Quartz Thermometer type 2804A. A cathetometer was used to monitor the level of mercury in the capillary. Experiments were conducted by filling the dilatometer at 60° with the amylose solution (52 g). The sample-arm tap was closed and the dilatometer was placed in the water bath at 32°. After 20 min, a small positive pressure, sufficient to introduce mercury into the capillary tube, was applied to the other side-arm and its tap closed. The level of mercury in the capillary tube was measured as a function of time.

Although barometric pressure was monitored, no compressibility corrections were made, as the correction was <1% of the observed volume change.

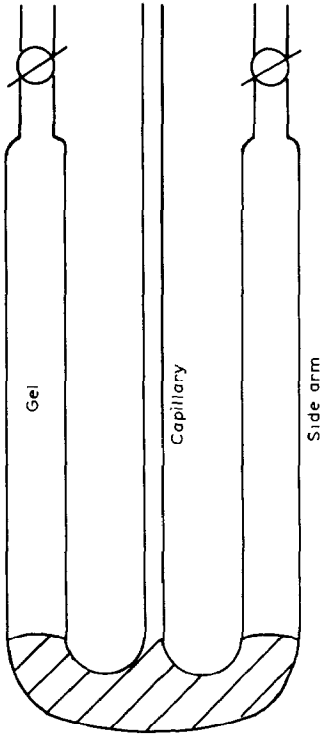

Fig. 1. Schematic diagram of dilatometer.

The apparatus was tested by studies of the isothermal volume change on gelation of gelatin[20]. The sign of the volume change agreed with that reported by Flory and Garrett[21]. Slight differences in the magnitudes of the volume changes reported in refs. 20 and 21 may be attributed to differences in the nature of the solvent used by the two sets of workers.

X-Ray diffraction. — Initial X-ray studies were made by using a wide-angle flat-plate camera. Amylose gels were prepared in thin-walled quartz capillaries, and, after storage for 3 h, powder-type diffraction patterns were recorded with exposure times of ~1.5 h. In order to follow the development of crystallinity with time, changes in the 100 diffraction maximum were measured using an Anton Paar K.G. Kratky small-angle camera fitted with a proportional detector. Amylose solutions were prepared in quartz capillaries and cooled from 65 to 32° within this camera. Intensity measurements were made at 10 (19 at higher concentrations and longer times) angular positions in the range of 2θ, the diffracting angle, from 0.0453 to 0.1353 radian, which includes the 100 reflection. The time from quenching at which each measurement was made was also recorded. Scans over this angular range were repeated during 8 days. Initial scans required 15 min each to record, but the time allocated to each scan was increased, to improve the precision of the measurements, as the observed rate of increase in intensity of the 100 reflection

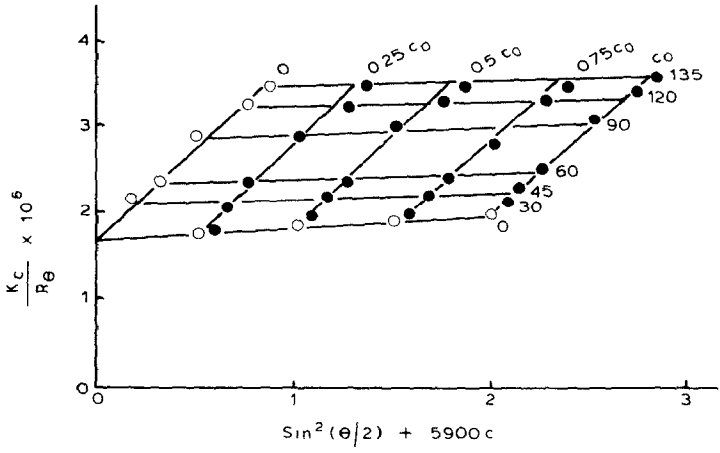

Fig. 2. Zimm plot for the amylose used in the study at 24°: wavelength, 442 nm; $C_0 = 0.34$ mg.mL^{-1}.

decreased. A scan was also recorded for the quartz capillary filled with water. These intensities were subtracted from each sample scan. Using the data, isochronal plots of intensity against scattering angle at fixed, elasped times were constructed. From these plots, the diffracted intensity of the 100 reflection above the background of the particle scattering intensity was determined at a series of fixed times and used as a measure of relative crystallinity.

RESULTS AND DISCUSSION

The fraction of amylose used for the gelation experiments was a linear amylose of high purity. Light-scattering studies demonstrated that true, dilute molecular solutions may be obtained *via* regeneration of the complex with 1-butanol. True solution behaviour was indicated by two experimental observations. Firstly, the samples showed no abnormally high light-scattering at low angles. Contamination with retrograded amylose is notorious for producing abnormal scattering at low scattering angles[20,22,23]. Secondly, as retrograded amylose is not readily attacked by alpha-amylase, digestion with alpha amylase would have resulted in excess scattering relative to the solvent at low scattering angles if retrograded amylose had been present. The residual scattering following digestion with alpha-amylase was found to be virtually identical to the solvent scattering at all angles. A typical Zimm plot obtained for an amylose solution is shown in Fig. 2. The purified amylose had a weight-average molecular weight of 500,000 and a z-average square radius-of-gyration of 2.5×10^{-16} m^2.

The behaviour of the specific viscosity (η_{sp}) as a function of concentration at 65° is shown in Fig. 3. At this temperature, retrogradation, as indicated by an increased turbidity, did not occur. At low concentrations, η_{sp} depended linearly on the amylose concentration. At higher concentrations, a marked change in con-

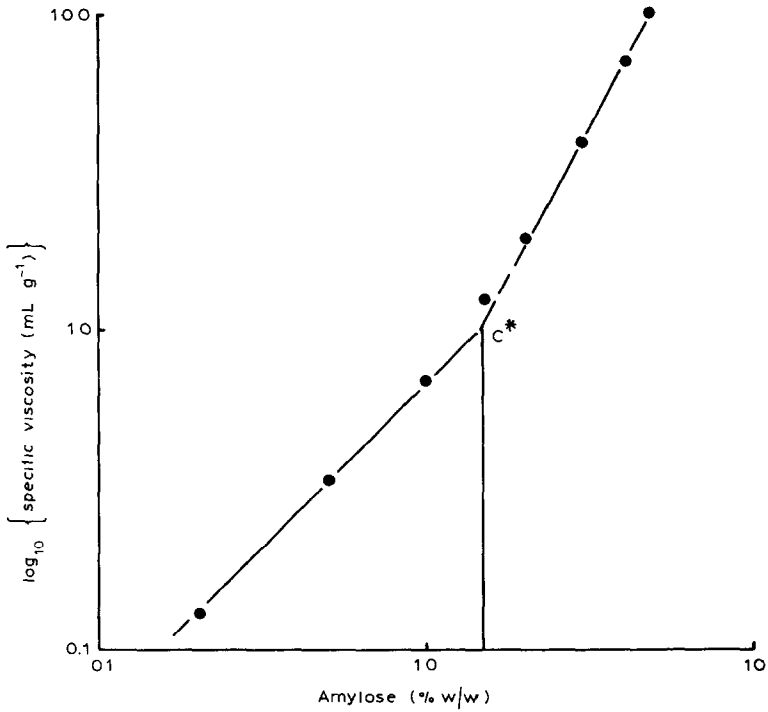

Fig. 3. Double logarithmic plot of specific viscosity *versus* concentration for amylose at 60° in aqueous solution.

centration dependence was observed. The specific viscosity was no longer simply dependent on the hydrodynamic volume of the polymer. The concentration C*, as defined in Fig. 3, may be regarded as the so-called overlap or entanglement concentration[24]. Above C*, η_{sp} shows a dependence on C^2. However, the present studies were carried out using Ubbelohde viscometers. Above C*, marked shear-thinning behaviour may occur and it would be necessary to extrapolate results to zero shear-rate in order to determine the exact nature of the η_{sp}–C dependence. The concentration at which the polymer molecules should completely occupy the available volume in solution may be calculated[24] in terms of C* and $[\eta]$, using the Flory–Fox expression for the intrinsic viscosity. An order of magnitude prediction suggests C* $[\eta] = 1.5$. For the amylose preparation ($\bar{M}_w = 500{,}000$), the present results suggest C* $[\eta] = 1.2$. In view of the polydisperse nature of the sample, the agreement is sufficiently good to justify attributing the change in concentration dependence occurring in the region of C* to polymer–polymer entanglement.

For polymer concentrations above C*, gelation occurred on cooling whereas, for concentrations less than C*, precipitation of amylose tended to occur on cooling. The concentration C* is difficult to define precisely experimentally. Similarly, the concentration C_0 separating gelation and precipitation on cooling is equally difficult to define exactly. It depends on the exact criteria and experimental

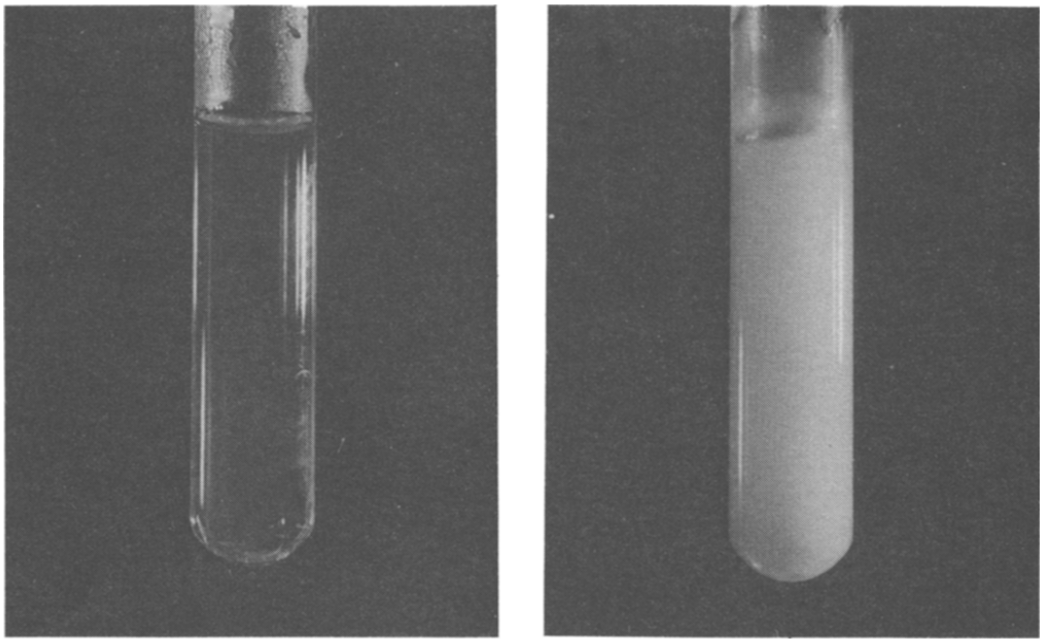

Fig. 4. Development of turbidity as a function of time for a 7% w/w amylose solution quenched to 32° (*a*) at zero time and (*b*) after 3 min at 32°

method of defining the existence of weak gels. However, it is clear that $C_0 \sim C^*$ and also that gelation may, in general, be considered to occur from an entangled solution on cooling. Thus, polymer entanglement is important in understanding the gelation of amylose. Measurements of the intrinsic viscosity, $[\eta]$, through the use of the relation $C^* [\eta] = 1.5$, may be used to estimate the value of C_0 for a particular sample of amylose.

A characteristic of amylose gels is their opacity (Fig. 4). The rate of development of opacity depends on polymer concentration and polymer molecular weight. At a given molecular weight, the rate at which the samples become turbid decreases with decreasing concentration of polymer. Fig. 5 shows results obtained for a 2.4% sample. The increase in turbidity suggests a change in the refractive index distribution and, thus, the density distribution within the sample. The change is consistent with a phase separation into polymer-rich and polymer-deficient regions. Light-scattering studies of the early stages of phase separation might permit an analysis of the mechanisms of phase separation (spinodal or nucleation and growth). However, the development of a gel structure suggests the establishment of a three-dimensional network of polymer-rich phase throughout the sample. Quantitative analysis of the present data is difficult, because such experimental problems as multiple scattering are likely to affect the observed turbidity at quite early stages of gelation.

Dilatometry has been employed to study phase transitions in polymer-diluent

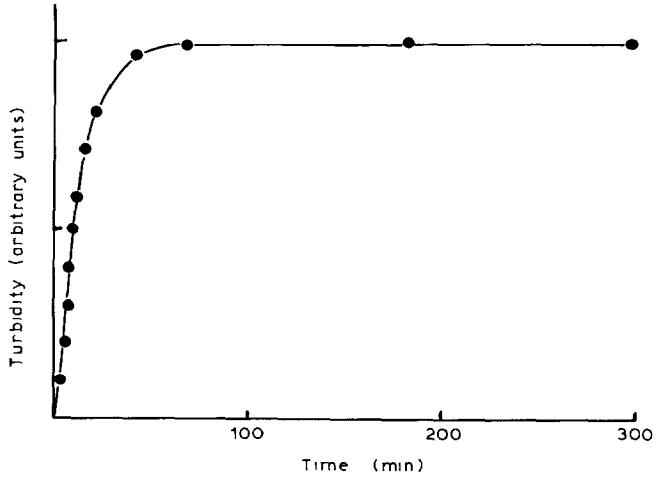

Fig. 5. Graph of turbidity *versus* time for a 2.4% w/w amylose solution on quenching to 32°.

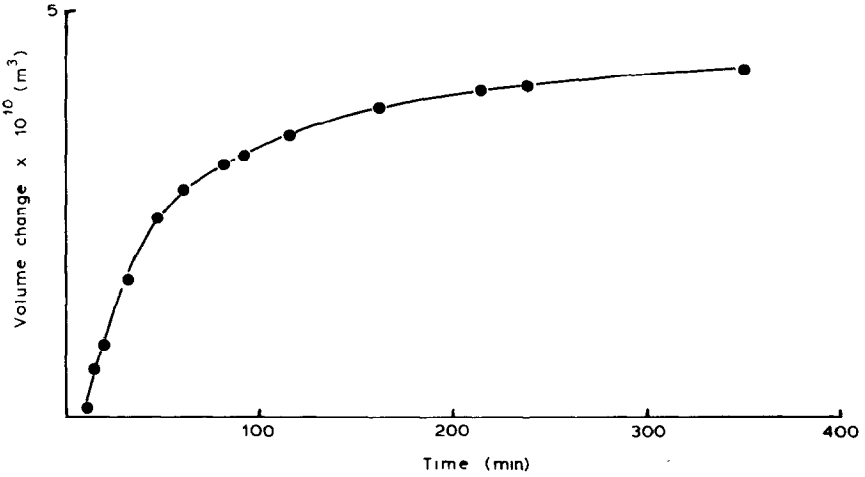

Fig. 6. Graph of volume change *versus* time for a 2,4% w/w amylose solution on quenching to 32°.

systems[21,25]. The measured volume changes reflect changes in the strength, number, and symmetry of the interactions between molecules. The volume change is directly related to the amount of polymer separating from solution. Fig. 6 shows the volume change (ΔV) observed on gelation of a 2.4% amylose sample. The positive volume change observed on demixing is worthy of comment. Crystallisation of polymers from the melt is normally accompanied by a negative volume change as polymer segments become aligned. Solute–solvent interactions are important for polymer separation from solution. Carbohydrate–water mixtures generally show a negative excess volume of mixing[26]. The magnitude of this change primarily depends on the strength and number of solute–solvent hydrogen-bonds. Thus, separation of the

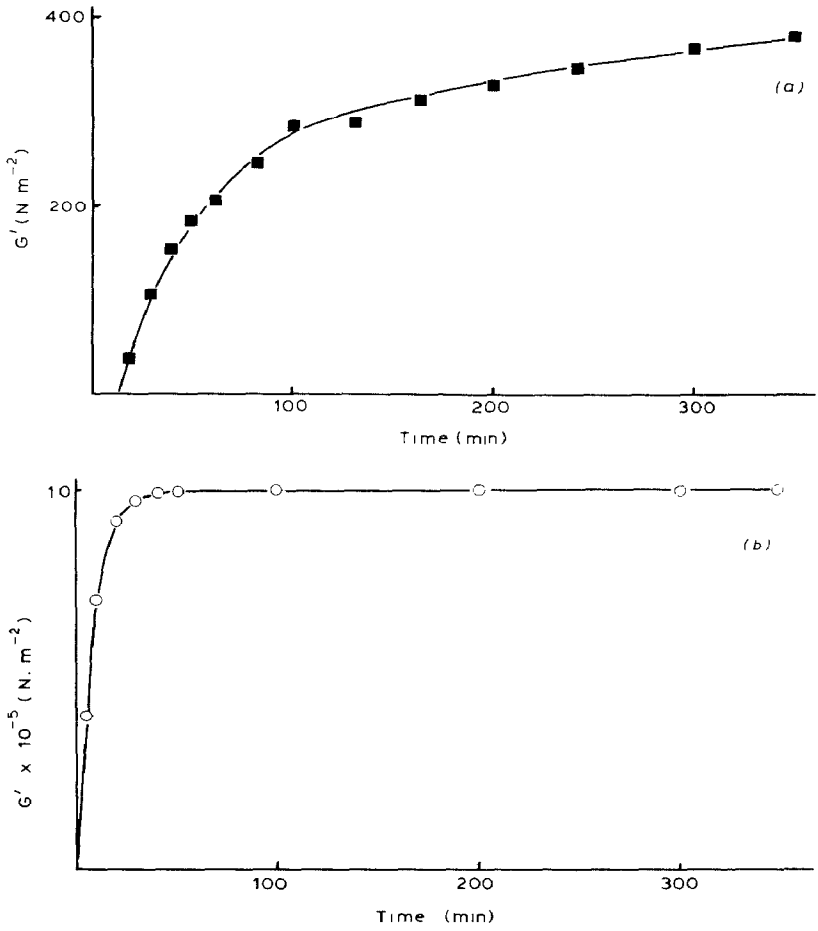

Fig. 7. Development of shear modulus, G', with time for (*a*) 2.4% and (*b*) 7% w/w amylose solutions on quenching to 32°.

solute from solution might be expected to lead to positive volume changes on demixing. Further information about the negative excess volumes of mixing of starch oligosaccharides would be needed if the present data are to be interpreted in terms of the amount of amylose contained in the polymer-rich phase.

The observed volume change is related to the extent of polymer separation from solution. Measurements of G' may be used to study the development of the network structure of the gel. Fig. 7 shows the development of the storage modulus as a function of time for 2.4% and 7% amylose samples. These data demonstrate several points. Firstly, the rate of development of G' is concentration-dependent. The curves of G' against t (time) cannot be scaled simply by multiplying G' by a suitable constant [*i.e.*, $K_G G'(t)$]. Secondly, after a sufficiently long period of time, the modulus appears to attain a saturation value G'_∞ which is dependent on con-

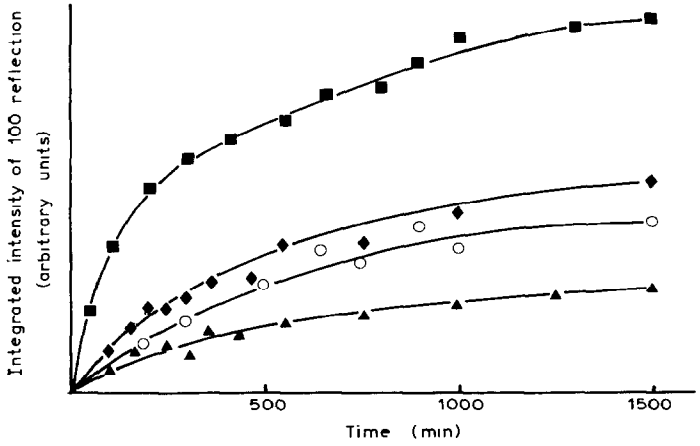

Fig. 8. Plot of development of crystallinity, expressed as the integrated intensity of the 100 peak, with time for (*a*) 2.4% (▲), (*b*) 3.5% (○), (*c*) 5% (◆), and (*d*) 7% w/w (■) amylose solutions at 32°.

centration. The value of G'_∞ obtained at 200 Hz agrees, to within ±10%, with values measured under static loading conditions[20]. The saturation values G'_∞ are strongly concentration-dependent. Experimentally, it was observed[20] that $G' \propto C^7$ in the concentration range 1.5–7%. However, it should be noted that values of G′ and G'_∞ are sensitive to the thermal history of the amylose sample[20]. Rates of quenching and the quenching temperature significantly affect the values of G′ and G'_∞. For example, the modulus G'_∞ of rapidly cooled gels is some 30% less than for slowly cooled gels (~4 h to reach maturation temperature). With suitable scaling, curves of ΔV–t and G′–t, for gels of a given concentration prepared under identical conditions, can be superimposed. This suggests that the development of the network structure depends directly on the phase separation of the amylose. It is interesting to note that the phase separation cannot be reversed by heating to 100°.

X-Ray diffraction data provide a direct measure of crystallisation within the amylose gel during gelation. Fig. 8 shows the development of crystallinity, monitored in terms of the area under the 100 peak (A), as a function of elapsed time following gelation. At a given concentration of amylose, the rate of development of crystallinity is substantially lower than the development of the turbidity, the volume change, and the shear modulus. Quite clearly, the rate at which crystallinity develops is markedly lower than the development of the network structure. Further, when the shear modulus has achieved a "saturation value" G'_∞, the crystallinity continues to increase with time. The rate of development of crystallinity appears to be independent of the polymer concentration. The curves in Fig. 8 may be normalised in the form K_A A(t) by appropriate choice of the constants K_A. If it is assumed that crystallisation is dependent on the local concentration of polymer, this suggests that the local concentrations within the polymer-rich and polymer-deficient regions each remain constant. Presumably, varying the overall amylose

concentrations alters the volume fraction of polymer-rich regions. This would also be consistent with the result that, whereas the rate of crystallisation is independent of concentration, the final level of crystallisation is concentration-dependent.

CONCLUSIONS

Viscosity measurements suggest that polymer–polymer entanglement occurs at a concentration C*. Amylose solutions are inherently unstable at room temperature, and precipitation is observed at concentrations <C*. At polymer concentrations >C*, opaque gels are formed on cooling hot, aqueous solutions. The development of opacity appears to be related to the irreversible phase separation into polymer-rich and polymer-deficient regions. The development of a phase-separated system and the development of the network structure of the gel are strongly correlated. This suggests that, for concentrations >C*, phase separations result in a continuous network of polymer-rich phase. The local composition of the polymer-rich phase appears to be independent of overall polymer concentration in the concentration range studied. The development of crystallinity, presumably within the polymer-rich phase, has been observed to develop slowly with time. There appears to be no direct relation between the kinetics of network formation and crystallisation. Gelation, or the early stages of retrogradation, appears to involve phase separation followed by a slow crystallisation in the polymer-rich phase. Factors which remain to be determined are the mechanism of phase separation, and quantitative estimates of the amount of phase-separated polymer and of the actual fraction of polymer present in crystalline regions at various stages of network formation. The present studies of amylose gels need to be correlated with direct studies of starch gels.

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